# $^{32}$P Analysis of DNA Adducts in Tissues of Benzene-Treated Rats

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Solid tumors have been reported in the Zymbal gland, oral and nasal cavities, liver, and mammary gland of Sprague-Dawley rats following chronic, high-dose administration of benzene. The carcinogenic activity of benzene is thought to be caused by activation to toxic metabolites that can interact with DNA, forming covalent adducts. A nuclease $P_1$-enhanced $^{32}$P-postlabeling assay, having a sensitivity limit of 1 adduct in $10^{9-10}$ DNA nucleotides, was found suitable for measuring aromatic DNA adducts derived in vitro from catechol, benzenetriol (BT), phenol, hydroquinone (HQ), and benzoquinone (BQ), potential metabolites of benzene. When DNA specimens isolated from tissues of female Sprague-Dawley rats at 24 hr after an oral gavage dose of 200 to 500 mg/kg, 5 days/week, in olive oil (3 mL/kg) for 1 day, 1 week, 5 weeks, and 10 weeks were analyzed by the $^{32}$P-postlabeling procedure, no aromatic adducts were detected unequivocally with DNA samples of liver, kidney, bone marrow, and mammary gland. With Zymbal gland DNA, three weak spots at levels totaling four lesions per $10^9$ DNA nucleotides were seen only after 10 weeks of treatment, and these adducts did not correspond chromatographically to major adducts in vitro from the above specified compounds. Consequently, this finding requires confirmatory experiments. This distinct adduct pattern may relate to tumor induction in this organ following benzene administration. Our results also indicate that DNA adducts derived from catechol, BT, phenol, HQ, and BQ are either not formed in vivo with benzene or formed at levels below the detection limit of 1 adduct per $10^{9-10}$ DNA nucleotides.

## Introduction

Benzene is a chemical intermediate used for the synthesis of pesticides, dyes, and plastic resins, a component of commercial gasoline, and is present in air, food, feed, tobacco, and pyrolysis products (1). According to the investigations of Maltoni et al. (2) and the National Toxicology Program (3), the chronic, high-dose administration of benzene to rats produces a variety of tumors, including Zymbal gland carcinomas, oral cavity carcinomas, hepatocarcinomas, and possibly, mammary carcinomas.

Studies on the mechanisms of carcinogenic action of benzene have focused on its conversion to toxic metabolites and its ability to damage DNA. Evidence suggests that benzene is hydroxylated in vivo to phenol and catechol (4,5). Phenol, which is the predominant product, can be further converted via a hydroquinone (HQ) intermediate to semiquinone and benzoquinone (BQ). Catechol may form either 1,2,4-benzenetriol (BT), which can be oxidized to its semiquinone and quinone species, or to the ring-opened product, trans,trans-muconaldehyde (4–6).

Semiquinones and quinones are generally considered to be toxic metabolites capable of interacting with DNA (7,8), but the genotoxicity of muconaldehyde, a hematotoxin (9), has yet to be elucidated.

BQ and HQ have been shown, by the application of HPLC and physical methods (7), to form adducts in vitro with calf thymus DNA, deoxyguanosine (dG), and deoxyadenosine (dA). The major adduct formed by the reaction of dG with BQ has been characterized as (3'OH) benzetheno($N$1,$N^2$)deoxyguanosine (7). In addition to BQ and HQ, phenol, catechol, BT, and $^{14}$C-benzene, have been shown to form adducts in vitro with DNA in the liver and in bone marrow mitoplasts prelabeled with $^3$H-dGTP or $^3$H-dATP, as determined by the profiling of DNA hydrolysate on a Sephadex LH-20 column (8). Seven guanine and two adenine adducts have been detected with benzene in this system (8). The question as to whether adducts similar to the in vitro products are generated in vivo has not been addressed, although administration of $^{14}$C- or $^3$H-benzene to rats and mice has been shown to cause incorporation of low-level radioactivity into DNA of liver, kidney, spleen, and bone marrow (10,11). In the present studies, we have addressed this question using a highly sensitive nuclease $P_1$-enhanced $^{32}$P-postlabeling assay (12,13), which allows the detection of aromatic and

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bulky nonaromatic adducts at a frequency as low as 1 modification per $10^{9-10}$ DNA nucleotides. Our results show that *in vitro* adducts generated with the above aromatic benzene metabolites are not formed *in vivo* at detectable levels in DNA samples isolated from the liver, kidney, and mammary gland of rats given benzene for 10 weeks. DNA from the Zymbal gland, however, showed three adducts, which appear not to correspond to the *in vitro* adducts from aromatic metabolites.

## Materials and Methods

The sources of chemicals and biochemicals have been given in the accompanying paper (14), except where specified below.

To validate the suitability of the $^{32}$P-postlabeling assay for benzene-type adducts, BQ-modified DNA (BQ-DNA) was prepared by incubation of rat kidney DNA (3 mM DNA-P) with BQ (9 mM, Aldrich Chemical Co.) at 37°C for 12 hr, followed by precipitation with ethanol. Control was carried out without the addition of BQ or DNA. To reduce the extent of adduction, BQ-DNA was mixed with unadducted DNA at the ratio of 1 to 300.

Female Sprague-Dawley rats, 4 months of age and weighing about 280 g (Charles River Laboratories, Raleigh, NC), were maintained on standard laboratory diet and water *ad libitum*. To prepare *in vivo* benzene-DNA samples, groups of rats (4 per group) were given, by oral gavage, 200 or 500 mg/kg benzene in olive oil (3 mL/kg) every day, 5 days/week for 1 day, 1 week, 5 weeks, or 10 weeks; the dose was switched from 500 to 200 mg/kg at week 4 to reduce rat mortality from benzene toxicity. The rats were sacrificed at 24 hr after dosing, and the following tissues were collected: liver, kidney, mammary gland, bone marrow (femur), and Zymbal gland. DNA was isolated from the tissues by a modified digestion and extraction procedure as described previously (15), except that the volumes of reagents were reduced 3-fold in the case of Zymbal glands. DNA modified *in vivo* with 2-acetylaminofluorene (AAF), used as a positive control, was prepared from the Zymbal glands of rats at 24 hr after a daily oral dosing of 40 mg/kg AAF for 5 days (14). The detection and quantitation of adducts in DNA samples (15 µg each) were accomplished by a nuclease $P_1$-enhanced $^{32}$P-postlabeling assay (12,13) coupled with TLC performed according to method A in the accompanying paper (14). $^{32}$P-Labeling of adducts without exposure of DNA digests to nuclease $P_1$, i.e., the standard procedure, was according to previous conditions (12,16).

## Results

### Validation of Nuclease $P_1$ Version of the $^{32}$P-Postlabeling Assay

Detection of aromatic DNA adducts by $^{32}$P-postlabeling can be enhanced three orders of magnitude by the pretreatment of DNA digests with nuclease $P_1$, which dephosphorylates normal nucleotides but not most aromatic adducted nucleotides (12,13). Since some adducted

nucleotides are, however, dephosphorylated with nuclease $P_1$, resulting in their low recovery or loss (12,13), it was necessary to validate the nuclease $P_1$-enhanced $^{32}$P-postlabeling assay for adducts derived from aromatic benzene metabolites.

The validation of the $^{32}$P-postlabeled adduct assay was carried out employing *in vitro* adducts. One major and several minor $^{32}$P-postlabeled adduct fractions were detected with *in vitro* BQ-DNA (Fig. 1B) but not with control DNA (Fig. 1A). These adducted nucleotides were found to be virtually resistant to 3'-dephosphorylation by nuclease $P_1$, since the adduct patterns, as well as adduct recoveries (data not shown), were similar with and without exposure (i.e., standard procedure) (Fig. 1B and 1D) to the enzyme. The nuclease $P_1$-mediated assay was, however, about three orders of magnitude more sensitive than the standard procedure, as evident from the observed enhancement of $^{32}$P incorporation into adducts (data not shown) and the approximately 500 times shorter film exposure required for adduct detection. Adduct 1 composed approximately 82% of total DNA modifications, which corresponded to 400 adducts per $10^7$ nucleotides. Reducing DNA modification about 300 times by dilution
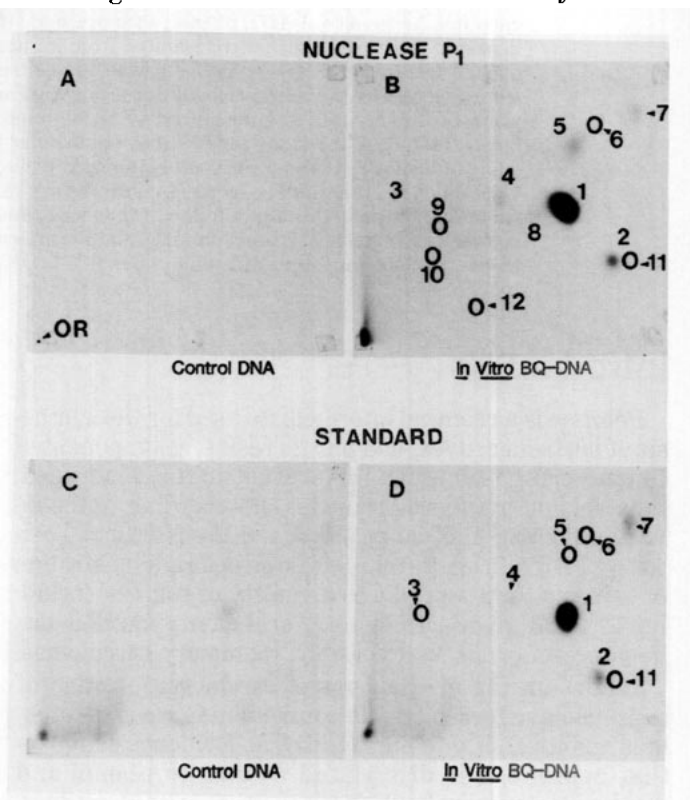


FIGURE 1. Comparison of nuclease $P_1$ and standard assays for BQ-DNA adducts. Autoradiograms of $^{32}$P-maps of labeled digests prepared from the indicated DNA samples with (A, B) and without (C, D) exposure to nuclease $P_1$. DNA specimens (15 µg) were digested and labeled under standard and nuclease $P_1$ conditions (12). Labeled adducts were purified, transferred *in situ* (23), and resolved by PEI-cellulose TLC *according to method A (14)*, except that the development in D3 solvent (bottom to top) was to 12.5 cm. Autoradiography was performed at 23°C for 30 min (A,B) and at −80°C for 2 days (C,D). Weak spots have been circled. OR, origin. The sensitivity of X-ray film is 4 to 5 times higher at -80°C than at 23°C.

with unadducted kidney DNA still permitted the detection of BQ adducts, but an increase in background radioactivity was noticed both in control and treated samples (Fig. 2). The background spots, some of which correspond to endogenous adducts (I-compounds) (17), reduced the sensitivity of detection for adducts of interest. The limit of detection of BQ-specific adducts varied from 1 to 10 adducts per $10^{10}$ DNA nucleotides, depending on the position of an adduct in relation to endogenous adducts. For example, adduct 3 could be detected at a higher sensitivity than adduct 1 (Fig. 2), which in turn was detected at a greater sensitivity than adduct 2.

Using the $^{32}$P-postlabeling assay, we detected a major product chromatographically identical to adduct 1 (Fig. 1A and 1B) when deoxyguanosine 3'-monophosphate was reacted with BQ (data not shown), showing that adduct 1 is a guanine derivative. The reaction of BQ with dG has been shown to generate (3'OH)benzetheno($N1,N^2$)dG as the major derivative by HPLC and NMR analyses (7).

These results, as well as the finding that DNA adducts are readily detectable in a dose-related fashion in Zymbal glands treated in culture with phenol, HQ, BQ, catechol, and BT (14), indicated that the nuclease $P_1$ version of $^{32}$P-postlabeling assay is applicable for the sensitive measurement of adducts derived from these benzene metabolites.

### Analysis of *In Vivo* Adducts

The validated nuclease $P_1$-mediated $^{32}$P-postlabeling procedure was applied to the detection of adducts in DNA samples isolated from tissues at 24 hr after a daily oral gavage dose of 200 to 500 mg/kg benzene for 1 day, 1 week, 5 weeks, or 10 weeks. No aromatic adducts were detected unequivocally in the liver, kidney, and mammary gland at any of the time points, while the Zymbal



Control DNA          In Vitro BQ–DNA (1:300 dilution)

FIGURE 2. Effect of lowering of DNA modifications on the $^{32}$P-postlabeling assay's sensitivity. *In vitro* BQ-DNA was diluted 300-fold with unadducted kidney DNA and was analyzed by the nuclease $P_1$ method. See the legend of Fig. 1 for additional details. Note that chromatographic development in the vertical direction was longer (20 cm) than that performed for maps shown in Fig. 1. Autoradiography was at –80°C for 15 hr. A, B, and C indicate reference background spots.

glands showed adducts only after 10 weeks of treatment. These findings are illustrated in Figure 3 with $^{32}$P-maps derived from 5-week and 10-week DNA samples. For liver, kidney, and mammary glands, $^{32}$P fingerprints of 5-week treated DNA specimens were both qualitatively and quantitatively very similar to those obtained with the corresponding control samples, indicating that benzene failed to elicit aromatic adducts at detectable levels of 1 adduct in $10^9$ nucleotides. Certain extra spots marked by arrows were seen inconsistently in treated samples and were not detectable after 10 weeks of treatment (maps not shown). The $^{32}$P-map of Zymbal gland DNA after 5 weeks was also similar to that of control DNA, while the 10-week sample showed three extra spots indicative of adducts. Adduct levels corresponded to 4 lesions per $10^9$ DNA nucleotides. Some spots indicated by arrows, presumably endogenous adducts, were reduced after 10 weeks of benzene treatment. Bone marrow DNA isolated from rats treated with benzene for 1 day and 1 week failed to show adducts (maps not shown). Adducts were readily detectable with *in vitro* BQ-DNA and *in vivo* AAF-DNA from Zymbal glands, both being used in this study as positive controls.

## Discussion

Nuclease $P_1$-enhanced $^{32}$P-postlabeling analysis revealed no DNA adducts in the liver, kidney, bone marrow, and mammary gland of rats given an oral gavage dose of benzene for 1 day, 1 week, 5 weeks or 10 weeks. Since the $^{32}$P-postlabeling assay is suitable for detection of DNA adducts derived *in vitro* from phenol, HQ, BQ, catechol, and BT (Fig. 1) (14), it can be concluded that adducts from these aromatic metabolites are not formed *in vivo* following benzene administration or are formed below detectable levels of 1 adduct in $10^9$ DNA nucleotides, i.e., 0.003 fmole/$\mu$g DNA. It is conceivable that the aromatic metabolites formed from the metabolism of benzene *in vivo* (4,5) either undergo conjugation or bind to proteins in tissues—both are processes that can inhibit DNA adduct formation.

The postlabeling assay results are not in agreement with the direct labeling findings that show a low level of radioactivity incorporated into the tissue DNA samples following $^3$H- or $^{14}$C-benzene administration to rats (10,11). This discrepancy can, however, be explained if the *in vivo* adducts are predominantly nonaromatic and polar, as are those probably derived from the putative open-ring derivative, muconaldehyde (6). In contrast to direct labeling, the $^{32}$P-postlabeling assay, under the conditions described, does not allow the recovery of such adducts. The most likely steps in the procedure at which adduct loss occurs are nuclease $P_1$ treatment of DNA digests (16), which can cause 3'-dephosphorylation of small adducts (16), and the purification of adducts by PEI-cellulose TLC. However, no adducts were detected when several variations of postlabeling techniques were tried (data not shown). These included: a) labeling of adducts by standard (16,17) and intensification conditions (18),
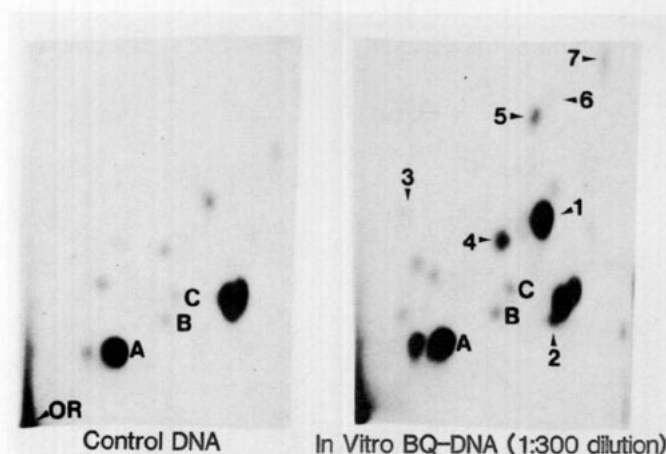
neither of which involves the exposure of DNA digests to nuclease $P_I$; b) the resolution of adducts by a combination of reverse-phase TLC and PEI-cellulose TLC (15,19,20), which allows the recovery of nonaromatic, bulky adducts (14); and c) the separation of adducts by two-dimensional PEI-cellulose TLC alone (16,21,22),

which facilitates the recovery of simple alkylated products. These variations of postlabeling are, however, three to five orders of magnitude less sensitive than the enhanced procedure using nuclease $P_1$ (12–14). Therefore, the enhancement of the $^{32}$P-postlabeling assay's sensitivity for small adducts by removal of unadducted nucleo-
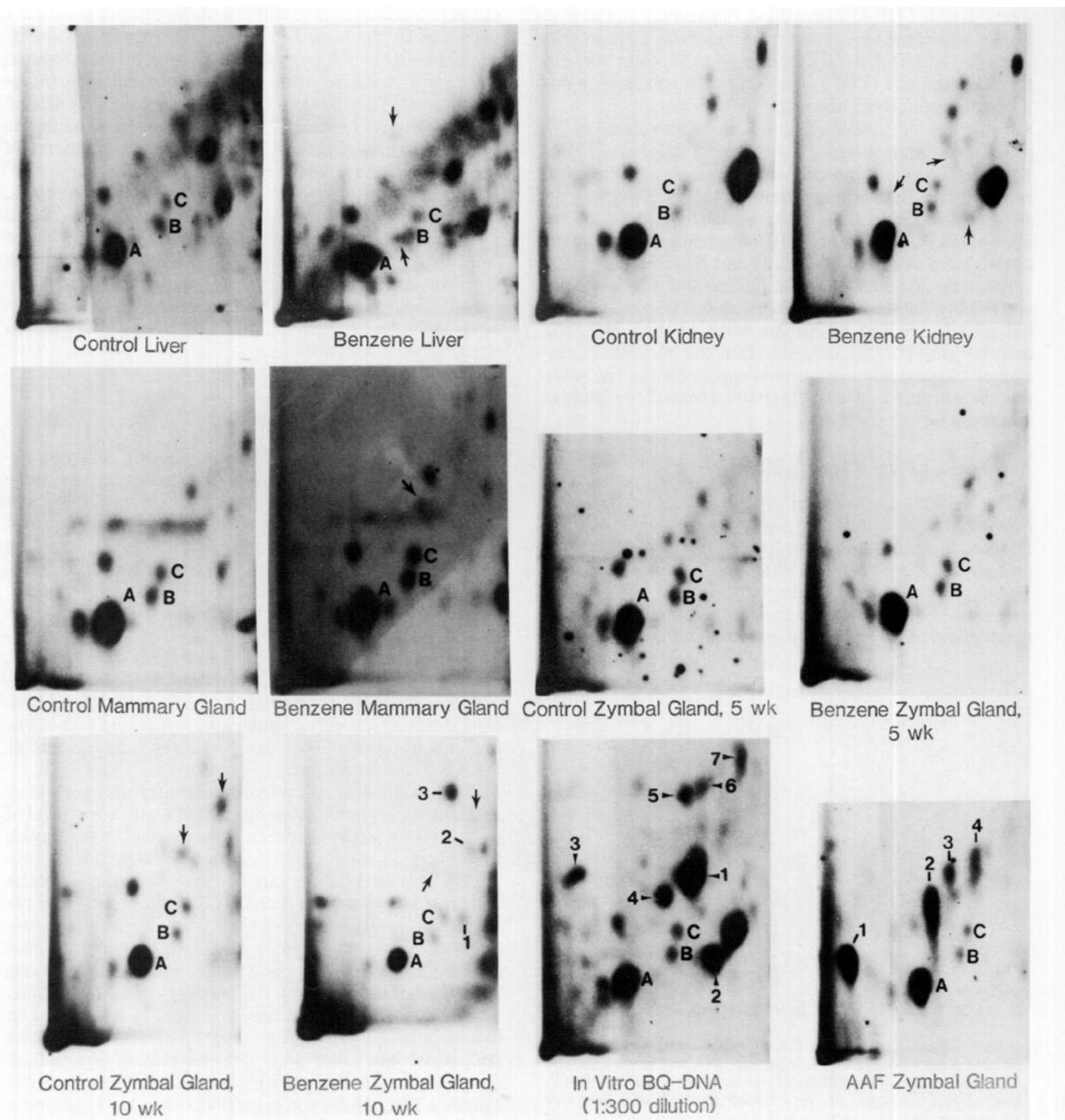


FIGURE 3. Autoradiograms of $^{32}$P-maps obtained with DNA samples isolated from the indicated control and benzene-treated tissues. Autoradiography was performed at −80°C for 48 hr (mammary glands) and 17 hr (others). A, B, and C indicate reference background spots. Adduct 1 seen with AAF-DNA has been identified previously as dpGp-C8-($N^2$-AF) and adduct 2 as dpGp-$N^2$-(C3-AAF) (17).

tides in DNA digests by HPLC prior to ³²P-labeling would probably assist in the detection of ring-opened derivatives presumably formed with ³H- or ¹⁴C-benzene *(10,11)*.

The detection of aromatic DNA adducts in Zymbal glands after 10 weeks of benzene treatment (Fig. 3) may relate to tumor formation in this organ *(2,3)*. These adducts apparently did not correspond chromatographically to the major adduct of BQ (Figs. 1 and 2) or to the major adducts generated by the interaction of phenol, HQ, BQ, catechol, and BT with DNA in Zymbal glands maintained in culture *(14)*. Adduct quantities from the *in vivo* Zymbal gland DNA are too low (∼ 0.01 fmole) to perform structural analysis by physicochemical methods, and thus, it is not possible to identify the benzene metabolite(s) present in the ³²P-postlabeled adducts. More data is needed to support the detection of *in vivo* adducts, since this result is from a single time point, i.e., 10 weeks (Fig. 3). Experiments indicating either the accumulation and persistence of the adducts upon benzene treatment for a period longer than 10 weeks or the formation of identical adducts in other target organs should provide such supportive data.

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